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453,352 No 4/1

From: Davis, Minh-Tam
Sent: Tuesday, July 01, 2003 9:47 AM
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Subject: Reprint request for 09/061417

- 1) Sussman, MA, 1998, Science: 281: 1690-1693. Please provide the month of the publication date for this reference.
- 2) Rowan, RA, 1990, Human Pathol, 21: 767-772.

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Pathologic Changes in the Long-term Transplanted Heart:

A Morphometric Study of Myocardial Hypertrophy, Vascularity, and Fibrosis

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Myocyte hypertrophy and myocardial fibrosis have been observed in transplanted human hearts, and both could potentially have an adverse effect on long-term cardiac function. There has been some concern that distant donor heart procurement and cyclosporine treatment increase the risk of these changes, but their incidence and severity have not been documented quantitatively in large numbers of cardiac transplant recipients. We used light microscopic morphometric methods to estimate myocardial collagen volume fraction and myocyte width in right ventricular endomyocardial biopsies from 95 recipients at 3 years posttransplantation, and electron microscopic stereology to estimate myocardial vascularity and myocyte myofibril content in 40 recipients, also at 3 years posttransplantation. We compared those with locally and distantly procured donor hearts (mean ischemic time 160 minutes) and cyclosporine versus noncyclosporine immunosuppression. Controls were pretransplant right ventricular biopsies from 20 donor hearts which were free of heart disease. We found no significant differences in myocardial collagen volume fractions. Myocyte hypertrophy was typical of all the transplant biopsies (mean myocyte width 20.2 μ m, SD 3.0 in all transplants versus 11.8 μ m, SD 2.2 in controls, $P < 0.001$), but distant donor procurement and cyclosporine had no significant effect. There were significant reductions of myofibril volume fraction in the transplants, which raises the possibility of gradual decompensation in some patients. There were no significant differences in myocardial vascularity, although a few patients were well below the control range. We conclude that distant donor heart procurement, with ischemic times averaging less than 3 hours, and cyclosporine treatment are not responsible for significant hypertrophy or fibrosis in most transplants. Hypertrophy is typical of the transplanted heart, and it is possible that associated abnormalities might have an effect on cardiac function in some long-term survivors. HUM PATHOL 21:767-772. © 1990 by W.B. Saunders Company.

Although cardiac transplantation provides effective rehabilitation for most recipients, structural and functional abnormalities are often found in cardiac allografts.¹⁻¹⁰ Chronic diastolic dysfunction ranges from subtle, occult restriction, which is common,¹⁻⁵ to clinically significant restrictive-constrictive physiology

in some patients.⁸ Myocardial fibrosis, which has been reported to be caused by cyclosporine, prolonged donor heart ischemic time, or rejection,^{9,11-13} has been implicated as a possible cause^{3,14}; other studies, however, have raised doubts about the existence of significant fibrosis or its contribution to diastolic restriction.^{1,3,6} Quantitative studies of myocardial collagen should help put this issue in perspective.

Ventricular hypertrophy can occur in long-term cardiac allografts,^{10,15,16} and this is cause for concern because of the potential for increased risk of ischemia, infarction, and congestive failure.¹⁷ In some forms of hypertrophy, myocardial vascularity and, in turn, coronary vasodilator reserve, can be reduced.^{18,19} If these changes occur in cardiac allografts, there might be an adverse effect on long-term outcome, particularly in the presence of accelerated coronary artery disease. Chronic hypertrophy can also lead to gradual loss of contractile myofibrils and eventual decompensation.²⁰⁻²²

The purpose of this morphometric study was to establish the incidence and severity of hypertrophy and fibrosis in right ventricular endomyocardial biopsies at 3 years posttransplantation, and to assess the influence of cyclosporine treatment and donor heart ischemic time. We used light microscopic morphometry for myocyte hypertrophy and interstitial collagen content, and electron microscopic stereology to assess myocardial vascularity and myocyte content of myofibrils and mitochondria.

METHODS

Light Microscopic Morphometry

Endomyocardial biopsies from the right side of the interventricular septum from a total of 95 cardiac transplant recipients, all at 3 years posttransplantation, were studied. The biopsies were divided into four groups, according to donor heart ischemic time and the type of immunosuppressive therapy the patient received:

1. Donor hearts procured locally (mean ischemic time 47 minutes, SD 6.0), and noncyclosporine immunosuppression (azathioprine and prednisone), $n = 25$.
2. Distantly procured donor hearts (mean ischemic time 162 minutes, SD 19) and noncyclosporine immunosuppression, $n = 20$.
3. Locally procured donor hearts (mean ischemic time 55 minutes, SD 10) and cyclosporine immunosuppression, $n = 25$.
4. Distantly procured hearts (mean ischemic time 156 minutes, SD 31) and cyclosporine immunosuppression, $n = 25$.

From the Department of Pathology, Stanford University School of Medicine, Stanford, CA. Accepted for publication November 14, 1989.

Supported by National Institutes of Health Grant No. HL13108.

Key words: endomyocardial biopsies, light and electron microscopic morphometry.

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0046-8177/90/2107-0012\$5.00/0

The number of rejection episodes for each patient during the 3 years was determined as reported previously.²³ A rejection episode was defined as biopsy evidence of rejection, extending from the first appearance of rejection to a subsequent diagnosis of resolving or resolved rejection. Episodes were categorized as mild, moderate, or severe according to the most severe grade reached during the episode, and the numbers of each were recorded for each patient.

The control group consisted of pretransplant biopsies from the right side of the septum in 20 donor hearts which were free of heart disease (mean donor age 24 years, SD 7). Comparisons were made for all parameters between locally and distantly procured donor hearts.

Trichrome-stained paraffin sections of an average of three pieces of tissue per patient were analyzed using a Microcomp morphometric system (Southern Micro Instruments, Atlanta, GA). None of the biopsies analyzed for any parameter contained evidence of a prior biopsy site. Microscopic images were displayed on a color video monitor screen for two-dimensional computer-assisted morphometry, or stereology using test system overlays. Microscopic fields were sampled systematically, and the number of fields sampled for each parameter was chosen to ensure a coefficient of error (standard error/mean) of 5% or less. The study was done blind. The group to which the biopsy belonged was unknown at the time of analysis.

The following quantities were estimated:

1. Myocyte width was measured in longitudinally oriented cells in which a nucleus was visible. A single transverse measurement was made through the middle of the nucleus.

2. The volume fraction of interstitial collagen in the myocardium was estimated by point counting.^{24,25} Points on the test system grid lying over blue-stained collagen fibers were counted. Any collagen which was clearly part of the endocardium was excluded.

Electron Microscopic Stereology

Right ventricular biopsies from a total of 40 patients at 3 years posttransplantation were processed for electron microscopy by our routine methods.^{26,27} They were divided into four groups of 10 biopsies each:

1. Locally procured donor hearts (mean ischemic time 44 minutes, SD 5) and noncyclosporine immunosuppression.

2. Distantly procured donor hearts (mean ischemic time 149 minutes, SD 28) and noncyclosporine immunosuppression.

3. Locally procured donor hearts (mean ischemic time 57 minutes, SD 21) and cyclosporine immunosuppression.

4. Distantly procured donor hearts (mean ischemic time 155 minutes, SD 10) and cyclosporine immunosuppression.

Controls were pretransplant biopsies from 10 donor hearts, free of heart disease (mean donor age 25 years, SD 9).

Sections for electron microscopy were cut from five tissue blocks per biopsy, each block embedded with random orientation. Five electron micrographs were taken in each section, each one from the upper left corner of a different grid square. Negatives were projected at a final magnification of 4,000 \times on a stereologic test system.

The following parameters were estimated:

1. Volume fraction of myofibrils in myocytes, excluding the volume of the nuclei (P myofibrils/ P myocyte - P nuclei).

2. Volume fraction of mitochondria in myocytes (P mitochondria/ P myocyte - P nuclei).

3. Length per unit volume (L_v) of blood vessels in the myocardium, estimated by counting the number of vessel profiles per unit area (Q_v) in an unbiased counting frame,²⁵ and, assuming random orientation of sections,^{24,25} $L_v = 2Q_v$.

4. Volume fraction of myocytes in the myocardium (P myocyte/ P myocardium).

5. Myocyte volume per unit blood vessel length, estimated from myocyte volume fraction and blood vessel length per unit volume: $V_l = \text{myocyte } V_v / \text{blood vessel } L_v$.

Sarcomere lengths were measured by a method reported previously²⁶ so that control myocyte widths could be corrected for possible differences in degree of contraction in comparison with the transplants. Corrections of control values were made by assuming an ideal cylindrical shape for myocytes, and then calculating the expected cross-sectional area from the myocyte width observed by light microscopy. The calculated cross-sectional area for light microscopic data and the cross-sectional area estimated by electron microscopy were each multiplied by the observed control sarcomere length. The resultant myocyte volume per sarcomere was then divided by the sarcomere length observed in the transplant biopsies to give the corrected control cross-sectional area expected at a mean sarcomere length equal to that of the transplants. Corrected myocyte width was calculated from the corrected cross-sectional area for light microscopic data.

Statistical Analysis

Data were analyzed using an MSP statistics program for the IBM PC computer (Keller, Marsh, and Associates, San Luis Obispo, CA). Comparisons of the means of three or more groups were made by one-way analysis of variance. Specific comparisons between individual group means or combinations of group means were made using the Scheffe method. The level of significance chosen was $P = 0.05$. Relationships between variables were analyzed by linear regression.

RESULTS

The results of light microscopic morphometry are summarized in Table 1. Myocyte width was significantly greater in all transplant groups compared with controls. The mean widths were slightly greater in the two groups with distantly procured donor hearts, but a comparison by the Scheffe method between the combined means of the locally procured hearts and the combined means of the distantly procured hearts was not statistically significant. Similarly, a comparison of the two cyclosporine groups with the two noncyclosporine groups was not statistically significant.

Control sarcomere length was 1.12 μm and was 0.95 μm in the transplants. The corrected control myocyte width was 13.3 μm , SD 2.12, which was significantly different from the nearest transplant group (local donors, noncyclosporine), $P < 0.01$.

There were no significant differences in collagen volume fraction. The differences in ischemic times for locally and distantly procured donor hearts were highly significant ($P < 0.001$). No significant relationship between collagen volume fraction and ischemic

TABLE 1. Light Microscopic Morphometry

	1 Controls (n = 20)	2 Local Donors Noncyclosporine (n = 25)	3 Distant Donors Noncyclosporine (n = 20)	4 Local Donors Cyclosporine (n = 25)	5 Distant Donors Cyclosporine (n = 25)
Myocyte width (μm)	11.8 (2.2) 9.6-19.5	18.8 (2.4) 15.1-25.0	20.6 (3.0) 16.0-30.0	19.6 (2.7) 16.1-24.2	21.6 (3.6) 14.8-31.1
Statistical comparisons					
All five groups (one-way analysis of variance): $P < 0.01$					
Specific comparisons (Scheffé method):					
$P < 0.01 = 1$ versus 2, 1 versus 5					
$P < 0.05 = 2$ versus 5					
Not significant = 2 + 4 versus 3 + 5, 2 + 3 versus 4 + 5					
Myocardial collagen volume fraction (%)	3.8 (2.0) 0.6-7.6	3.8 (1.8) 1.0-7.5	4.9 (2.6) 1.2-9.8	4.7 (2.5) 0.9-11.0	4.5 (2.2) 1.7-9.0
Statistical comparisons					
All five groups (one-way analysis of variance): not significant					

Note: Data are means, with standard deviations in parentheses, and ranges.

time could be found by linear regression analysis in the four transplant groups combined.

Data on the numbers of rejection episodes seen in biopsies in the four groups during the prior 3-year period are summarized in Table 2. The two groups treated with cyclosporine had a significantly greater number of combined mild, moderate, and severe episodes, as well as a significantly greater number of moderate episodes than the noncyclosporine groups. The incidences were approximately two times and three times greater, respectively. There were no significant differences in the number of severe rejection episodes. When analyzed by linear regression, a positive relationship was found between collagen volume fraction and rejection episodes ($P < 0.01$). The correlation coefficient was 0.24.

Of the 95 biopsies (285 tissue pieces) analyzed by light microscopic morphometry, five biopsies had evidence of rejection in progress: two in the local donors, cyclosporine group, two in the distant donors,

cyclosporine group, and one in the distant donors, noncyclosporine group. Four of these were mild rejection, and one was moderate. In all five, morphometric parameters were near the means of their respective groups, so these biopsies were not excluded from the study.

The results of electron microscopic morphometry are summarized in Table 3. Myofibril volume fractions were significantly lower than in controls in three of the four transplant groups. There was no significant difference between local and distant donor hearts, but the patients treated with cyclosporine had a significant reduction compared with the noncyclosporine patients.

Mean mitochondrial volume fractions were reduced in the four transplant groups, but the only statistically significant difference was between controls and the local donors, cyclosporine group.

All of the transplant groups had lower mean myocardial blood vessel L_v than controls, and higher mean myocyte volume per unit blood vessel length than controls, but none of these differences reached statistical significance.

TABLE 2. Number of Prior Rejection Episodes

	Mild, Moderate and Severe Combined	Moderate	Severe
Local donors Noncyclosporine	2.12 (1.49)	0.84 (0.96)	0.11 (0.32)
Distant donors Noncyclosporine	2.13 (1.61)	0.81 (0.83)	0.13 (0.34)
Local donors Cyclosporine	3.81 (1.80)	2.40 (1.99)	0.07 (0.26)
Distant donors Cyclosporine	4.42 (2.01)	2.56 (1.98)	0.22 (0.43)
Statistical comparison (one-way analysis of variance)	$P < 0.01$	$P < 0.01$	NS

Note: Data are means with standard deviations in parentheses. Abbreviation: NS, not significant.

DISCUSSION

The results of this study indicated that myocyte hypertrophy was typical in right ventricular endomyocardial biopsies at 3 years after cardiac transplantation, regardless of ischemic time or differences in immunosuppressive treatment, and that myofibril volume fraction was often reduced. Overall myocardial vascularity, however, was normal in most transplants, but a few patients did have marked reductions. With regard to myocardial fibrosis, our results indicated that distant donor heart procurement with ischemic times averaging less than 3 hours, and cyclosporine immunosuppression did not cause a significant overall increase in myocardial collagen.

TABLE 3. Electron Microscopic Morphometry

	1 Controls n = 10	2 Local Donors Non-cyclosporine n = 10	3 Distant Donors Non-cyclosporine n = 10	4 Local Donors Cyclosporine n = 10	5 Distant Donors Cyclosporine n = 10
V_v myofibrils (% myocyte volume)	47.7 (2.9) 42.2-54.0	46.3 (3.6) 39.1-51.9	40.5 (2.3) 36.3-43.4	38.3 (2.8) 34.2-42.4	40.5 (4.2) 33.3-47.0
Statistical comparisons All five groups*: $P < 0.01$ Specific comparisons†: $P < 0.01 = 1$ versus 3, 4, and 5, 2 + 3 versus 4 + 5 Not significant = 1 versus 2, 2 + 4 versus 3 + 5					
V_v mitochondria (% myocyte volume)	25.3 (2.8) 20.1-29.2	21.4 (2.3) 16.3-24.2	23.6 (9.0) 14.4-27.9	17.8 (3.2) 13.3-22.4	20.1 (3.8) 13.0-28.1
Statistical comparisons All five groups*: $P < 0.025$ Specific comparisons†: $P < 0.05 = 1$ versus 4 Not significant = 1 versus 2, 1 versus 5, 2 + 3, 4 + 5, 2 + 4 versus 3 + 5					
L_v blood vessels mm/mm ³	1350 (298) 912-1670	1029 (310) 462-1404	958 (273) 462-1316	1058 (243) 738-1492	1013 (386) 614-1838
Statistical comparisons All five groups*: not significant					
μm^3 myocyte per μm blood vessel	608 (132) 456-792	866 (356) 498-1728	930 (400) 555-1836	716 (154) 429-908	837 (262) 397-1287
Statistical comparisons All five groups*: not significant					

Note: Data are means, with standard deviations in parentheses, and ranges.

* One-way analysis of variance.

† Scheffé method.

Again, however, there were a few biopsies with collagen volume fractions well above the control range. To establish whether or not there is any functional significance associated with the structural changes we found, precise correlations need to be made by individual cases.

The results of this study support the conclusions of our previous study¹⁰ that myocyte hypertrophy is common, if not universal, in cardiac allografts, and extend that finding to a larger sample of patients. In contrast to the previous study, however, we did not find a statistically significant difference between locally and distantly procured donor hearts, even though there was a tendency for greater hypertrophy in the distant donors, possibly as compensation for greater ischemic and reperfusion damage.²⁸ Also, there was no significant difference between cyclosporine and noncyclosporine groups, even though systemic arterial hypertension is prevalent in patients treated with cyclosporine.⁶

Differences in degree of contraction might affect measurements of myocyte width or cross-sectional area,²⁶ but correction of our data for differences between control and transplant sarcomere lengths showed that there was still highly significant evidence of hypertrophy in the transplants.

The overall right ventricular hypertrophy in all groups of transplant patients might have more than one cause. Probably the most likely is volume overload, since the denervated transplanted heart in-

creases cardiac output by the Frank-Starling mechanism, with increased end-diastolic volume.^{29,30} Right ventricular dilatation and wall thickening have been found early after transplantation and persisting at 1 year follow-up, apparently as a result of chronic volume overload.¹⁵ Pressure overload may also be involved in some patients, because elevated pulmonary artery pressure is common early after transplantation and may remain abnormally high for years in some cases.^{1,3,4,6} Even a mild pulmonary hypertension would increase ventricular wall stress significantly and provide a strong stimulus for hypertrophy if the right ventricle is dilated.³¹ A small contribution might also be made by compensatory hypertrophy as a result of ischemic and reperfusion damage. This is suggested by the tendency for greater hypertrophy in distantly procured donor hearts, even though the difference is small and not statistically significant.

The fact that myocardial vascularity remained normal in most patients is consistent with the possibility that hypertrophy was caused by a relatively mild volume overload in which the increase in myocyte mass is accompanied by angiogenesis,^{18,19,32,33} which maintains normal vascularity. In a few of the biopsies, however, the definite reduction of vascularity suggests that a small minority of cardiac transplant recipients may have pathologic hypertrophy due to pressure overload or severe volume overload in which the increase in myocyte mass is not accompanied by compensatory angiogenesis,^{18,19,32} resulting

in reduced coronary vasodilator reserve.^{18,19} Angiogenesis can be inhibited by steroids, so there is a possibility that patients with a mild volume overload had reduced vascularity because of immunosuppressive treatment; the presence of heparin appears to be necessary for this effect of steroids, however.³⁵ Reduced coronary reserve has been observed in cardiac transplant recipients with ventricular hypertrophy,³⁴ and, along with accelerated coronary artery disease, diminished vascularity should be considered as a possible contributing factor.

The significant reduction of myofibril volume fraction in the transplants is characteristic of severe or chronic hypertrophy²⁰⁻²² and has been associated with impaired ventricular pump function.^{21,22,36} In normal myocardial growth or physiologic hypertrophy, a normal myofibril volume fraction is maintained.³² The reduced myofibril volume fractions which we observed could have been the result either of insufficient synthesis of myofibrillar proteins during hypertrophy or a loss of myofibrils taking place after hypertrophy and myofibril synthesis had been completed. In either case, the total amount of myofibrillar protein in the heart could be greater than before hypertrophy of the donor heart, but if the volume fraction is reduced, the contractile force generated per unit mass could be less than normal.^{21,22,36} There is evidence for the latter possibility that an initially well-compensated heart may be weakened by gradual loss of myofibrils with time.²⁰ A gradual decline of cardiac index has been observed in cardiac transplant recipients over a period of years,³⁷ but there is no clear evidence that reduced contractility is involved in ventricular dysfunction in cardiac allografts.

The significant difference in myofibril volume fraction between the combined noncyclosporine and combined cyclosporine patients was not expected. The explanation might be that it is an effect of the relatively small sample size, rather than an effect of the type of immunosuppression. Without a significant difference in myocyte hypertrophy, there does not seem to be a reason for reduced myofibril content in patients treated with cyclosporine.

Our quantitative findings on myocardial collagen content support the conclusions of nonquantitative studies^{3,6} that in the majority of cardiac transplants, myocardial fibrosis is not significant. In some patients, however, endomyocardial biopsies do show definite fibrosis, which may have several potential causes. We found evidence of a relationship between collagen content and the number of previous rejection episodes, but the increase in collagen was small, and it does not appear that statistically significant scarring was caused in our study patients by their mostly mild and moderate rejection episodes.

There have been several reports that cyclosporine causes myocardial fibrosis,^{11-13,38,39} but these studies have not documented the incidence in human cardiac allografts or have indicated that a minority of recipients are affected. Our results support the view that there is not an obligatory fibrosis with cyclosporine

treatment, but do not rule out the possibility that there might be subtle increases in some patients, perhaps in those treated with higher doses.

Prolonged donor heart ischemia has been found to cause myocardial fibrosis in experimental animals,^{40,41} but in the patients in our study who received distantly procured donor hearts, with an average ischemic time of less than 3 hours, there was no significant overall fibrosis, and no linear relationships between collagen content and ischemic time. All of those in our study had a donor heart ischemic time less than 4 hours, but because 4 to 6 hours of ischemia has been associated with a higher early mortality rate,⁴² it seems possible that these longer periods of ischemia might cause greater myocardial damage leading to significant fibrosis. Also, small areas of fibrosis could be the result of localized ischemia due to microemboli,⁹ regardless of the ischemic time of the whole donor heart.

Precise clinicopathologic correlations are needed to establish whether or not diastolic abnormalities seen in cardiac allografts are due to myocardial fibrosis, or to any of a number of other possible causes. None of the patients in our study were among those who were found to have significant restrictive-constrictive diastolic abnormalities.⁸ The cause of their diastolic dysfunction remains unclear, and we are currently analyzing collagen volume fractions in biopsies from that group.

We conclude that myocyte hypertrophy is typical in transplanted human hearts at 3 years and is often accompanied by reduced myofibril volume fraction, which raises the question of possible gradual decompensation. We also conclude that distant donor heart procurement with an average ischemic time less than 3 hours does not cause an overall significant increase in myocyte hypertrophy or myocardial fibrosis. The same appears to be the case for cyclosporine treatment.

As the number of long-term survivors and the duration of their survival increase, continued evaluation of the structure and function of the transplanted heart may highlight factors which influence long-term success.

Acknowledgment. We are grateful for the contributions of Hae Rhi Lee, and to Graciela Rodriguez for preparation of the manuscript.

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